Characterization of Rat Hepatic Acetyltransferase

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Rat liver cytosol is capable of *N*-acetylation (NAT) of arylamines, *O*-acetylation (OAT) of arylhydroxylamines, and *N,O*-acetyltransfer (AHAT) of arylhydroxamic acids. Physical, enzymatic, and immunochemical techniques now support the conclusion that a single 32 kDa protein accounts for all of these activities. Of the five immunoglobulin (IgG_1) mouse monoclonal antibodies (mAb) produced against this protein, each affected one or more of these acetylation activities. When mixed with rat hepatic cytosol and then chromatographed on a gel filtration column, mAbs 1F2 and 5F8 increased the apparent size of all enzymes capable of acetylation from 32 kDa to the exclusion volume. Each of the mAbs reacted with only a single 32 kDa protein on SDS-PAGE/Western blots, regardless of the state of purity of the enzyme. This enzyme is unstable in low salt solutions, as reflected by a relative loss in NAT versus AHAT activity, but it does not result in changes in either molecular weight or isoelectric point (pI). A second form of instability is shown by the formation of more basic peptides with pIs as high as 6, again without change in molecular weight. Although NAT activity is retained in acetyltransferase (AT) that has a minimally modified pI, further increases in pI result in total loss of enzyme activity. The differential effects of the mAbs on AT suggest that the ratios of NAT, OAT, and AHAT may be highly dependent on the conformation of the enzyme and, consequently, provide insight as to why the abilities of ATs from different species exhibit such dissimilar potentials for the activation of aromatic amines by OAT and AHAT. — Environ Health Perspect 102(Suppl 6):91–93 (1994)

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Introduction

Exposure to aromatic amines comes from occupational, environmental, dietary, and pharmacologic sources. Experimentally, these compounds have been shown to have a multiplicity of carcinogenic, mutagenic, or toxic effects in a variety of biologic systems (*1*). Metabolic activation of these compounds involves *N*-oxidation followed by conjugation to form *O*-esterified hydroxylamines that can react with DNA (*2*). Reactive *N*-acetoxy metabolites can be formed through the direct *O*-acetylation (OAT) of hydroxylamines by acetyl-S-CoA (*3*), or through internal *N,O*-acetyltransfer of acetohydroxamic acids (AHAT) (*4,5*). These metabolites, which are capable of altering nucleic acid, are believed to be involved in the production of mammary and possibly other tumors by these agents (*6*).

The objective of this study was to establish the nature of the cytosolic enzyme(s) of rat liver that is capable of *N*-acetylation (NAT), OAT, and AHAT. The rat was selected for study because of the relatively greater susceptibilities of several organs of this species as compared, for example, to the mouse (*1*), which may be due to the greater ability of the rat to activate aromatic amines by formation of *N*-acetoxyarylamines (*7*). The results of this study support the conclusion that a single enzyme from rat liver cytosol is capable of NAT, OAT, and AHAT.

Acetylation Assays

Three types of assays were used to estimate acetylation capability: *a*) estimation of the *N*-acetylated product, *b*) estimation of DNA adduct formation, and *c*) loss of substrate. AHAT, OAT, and NAT assays were carried out as in Table 1. Except for localization of acetyltransferase (AT) in column fractions, all samples were assayed at six to 10 different enzyme concentrations. The activities were derived from an average of those concentrations that showed a linear response.

Effect of Buffer Concentration on Enzyme Activity

Rat liver was homogenized in varying concentrations of sodium pyrophosphate (NaPPi) buffer (1mM DTT, pH 7.4). After differential centrifugation, enzyme assays were carried out in 50 mM NaPPi buffer. As the buffer concentration was lowered from 50 to 2 mM, the ratio of 2-AF NAT:AHAT was lowered from 33 to 16. Assay of the acetylation activities following exposure to techniques that employed low buffer concentrations (e.g., DEAE ion exchange chromatography in 2 mM NaPPi), showed a similar change in the ratio of 2-AF NAT:AHAT. The ratios of AHAT:OAT:Booth:SMZNAT (1:3:0.05:30) were unaltered as was the ratio of 2-AF NAT:PABA NAT (1.5:1).

Enzyme Characterization Using Monclonal Antibodies

Five monoclonal (mAbs) were produced using a partially purified rat hepatic AT as an antigen. To assess their effects on AT activities, varying quantities of mAbs were added to enzyme preparations and incubated at 4°C under argon for 2 hr. Enzyme assays were performed using this mixture as the enzyme preparation. The degree of inhibition was calculated based on the decrease of activity compared to that of control incubations without mAb. Each of the mAbs was shown to affect one or more of the acetylation reactions. The mAbs fell into three groups: 1A3 inhibited all three acetylation reactions, 1F2 and 5F8 inhibited AHAT and OAT but increased 2-AF NAT, and 4B4 and 4B5 had no affect on AHAT or OAT but increased 2-AF NAT activity. Estimates of the abilities of these mAbs to complex with acetylases present in liver cytosol were obtained by mixing the mAbs with rat liver cytosol and then chromatographing the mixture on a gel filtration column. Experiments with 1F2 and 5F8 led to the elution of all AT activities from the column with the exclusion volume (i.e., >70 kDa) instead of their characteristic migration as a 32 kDa molecular weight protein. Thus, both of these mAbs bound all protein capable of acetylation. The apparently lower affinities of the 1A3, 4B4, and 4B5 mAbs failed to modify the elution pattern of the AT.

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Table 1. Reactions catalyzed by cytosolic rat liver acetyltransferase.

	Acetyl donor	Acetyl acceptor	Reference	Reaction	Detection
NAT	^{3}H-Acetyl-S-CoA	Arylamine		*N*-Acetylation	^{3}H-Acetamide
		2-AF	(*8*)		
		SMZ	(*9*)		
		PABA	(*8*)		
OAT	Acetyl-S-CoA	Arylhydroxylamine (ring labeled) N-OH-DMABP	(*8*)	*O*-Acetylation	^{3}H-Arylamine nucleic acid adduct
AHAT	Arylhydroxamic acid (ring labeled) N-OH-AAF	Arylhydroxylamine[a] (ring labeled) N-OH-AF	(*3,8*)	*N,O*-Acetyltransfer, intramolecular transfer	^{3}H-Arylamine nucleic acid adduct
Booth	Hydroxamic acid N-OH-AABP	Arylamine AAB	(*10*)	*N*-Acetylation (*N,N'*-transacetylation)	Loss of absorbance with loss of arylamine

Abbreviations: NAT, *N*-acetylation; OAT, *O*-acetylation; AHAT, *N,O*-acetyltransfer; N-OH-AAF, *N*-hydroxy-*N*-acetyl-2-aminofluorene; N-OH-AABP, *N*-hydroxy-*N*-acetyl-4-aminobiphenyl; 2-AF, 2-aminofluorene; SMZ, sulfamethazine; PABA, *p*-aminobenzoic acid; N-OH-DMABP, *N*-hydroxy-3,2'-dimethyl-4-aminobiphenyl; AAB, 4-aminoazobenzene. [a]Formed by deacetylation of the arylhydroxamic acid (N-OH-AAF).

The mAb showing the highest inhibition (1A3) was covalently linked to protein A Sepharose for use as an immunoaffinity absorbent. Application of an AT preparation, partially purified from cytosol by sequential ion exchange chromatography on DEAE and gel filtration chromatography, to this column resulted in over 90% of the AHAT activity emerging in the flow-through fractions (i.e., in 50 mM NaPPi at pH 7.4 [1 mM DTT]). SDS-PAGE of the fractions showed that the more slowly emerging AT was displaced from the other proteins and was purified to apparent homogeneity. This protein, which was immunoreactive on SDS-PAGE, had AHAT, OAT, NAT, and Booth activities. A second affinity column was produced using mAb 1F2. In contrast to the 1A3 column, the 1F2 column retained all AHAT, OAT, and NAT activity. Western blot analysis indicated that while the AT preparation applied to the column showed immunoreactivity, none of the antigen was detected on elution of the column with NaPPi buffer. On elution of the column with diethylamine, pH 11.2, SDS-PAGE of the eluted fractions showed a single, immunoreactive band at 32 kDa. Although this column had a high binding capacity, the enzyme activity was lost during the procedure.

The uniqueness of rat liver AT was further supported by the observations that only a single immunoreactive band at 32 kDa was obtained with each of the 5 mAbs, with preparations obtained sequentially by precipitation of cytosol with ammonium sulfate, ion-exchange chromatography, gel filtration, and both of the immunoaffinity columns described above.

Isoelectric Focusing

The isoelectric point (pI) of the protein was determined using an LKB Ultraphor. AT at various stages of purity were electrophoresed on a prefocused Ampholine polyacrylamide gel, pH 3.5 to 9.5. Western blot analysis showed a major immunoreactive band at pI 4.5 using an ammonium sulfate preparation (Figure 1). A minor band at pI 4.8 was observed inconsistently upon repetition of the procedure. As purification proceeded through the steps described above, additional bands with higher pIs were found. In an attempt to associate activity with pI, IEF in polyacrylamide gel of AT purified through gel filtration was sliced, the protein was eluted, and acetylation assays were performed. Two bands of activity were found. The protein at pI 4.5 had AHAT, Booth, OAT, PABA NAT, 2-AF NAT, and SMZ NAT activities, while the protein at pI 4.8 had only PABA NAT and 2-AF NAT activities. SDS-PAGE/Westerns of the eluted proteins showed only a single immunoreactive band at 32 kDa.

In an attempt to ascertain whether the enzymatically active component with the higher isoelectric point was native to rat liver or whether it had been formed as a consequence of laboratory manipulation, a rat liver preparation maintained in 50 mM

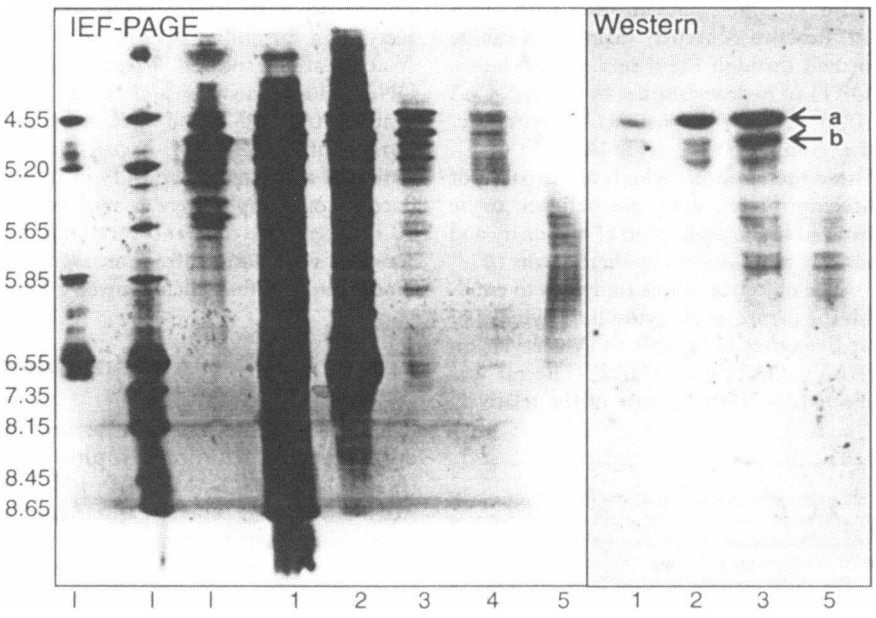

Figure 1. Analytical isoelectric focusing (IEF) of acetyltransferase at various stages of purification. IEF was carried out on IEF-PAGE at 10°C for 2 hr following purification by: lane 1, ammonium sulfate precipitation; lane 2, ion-exchange chromatography; lane 3, gel filtration; lane 4, 1A3-affinity chromatography; and lane 5, 1F2-affinity chromatography. Protein detection was by silver stain. Western blot analysis utilized a mixture of antiacetyltransferase mAbs. The lanes marked I contain isoelectric point standards. The immunoreactive proteins marked a and b correspond to the two peaks of enzyme activity described in the text.

NaPPi buffer through homogenization, differential centrifugation, gel filtration, and concentration by ultrafiltration under argon was focused in solution for 2 hr on a BioRad Rotofor in which a prefocused gradient had been established. The pH of the fractions was determined prior to mixing with an equal volume of 100 mM NaPPi buffer. Subsequent enzyme assays and SDS-PAGE/Westerns all showed a single peak of activity at pH 4.8/32 kDa with no evidence of heterogeneity.

Protein Analyses

The possibility that the loss of carbohydrate from AT might account for the formation of AT derivatives with increased pIs was examined by treating a partially purified AT (after DEAE ion exchange chromatography) preparation with neuraminidase. The reaction was followed with AHAT and 2-AF NAT assays and with SDS-PAGE/Western blot analysis. Neither loss of AHAT activity, as compared with 2-AF NAT activity, nor increases in pI were observed. AT was also tested for carbohydrate using an enzyme immunoassay system to detect digoxigenin-labeled glycans (*11*). Transferrin was used as a positive control. To localize AT, a Western blot of a duplicate gel was developed using a mixture of mAbs. Rat hepatic AT proved to be negative for glycan content.

Discussion

Rat liver cytosol is capable of NAT of arylamines, OAT of arylhydroxylamines, and AHAT of arylhydroxamic acids; the latter two reactions may be directly involved in the production of tumors by these agents (*6*). Physical, enzymatic, and immunochemical techniques now support the conclusion that a single 32 kDa protein is capable of all of these activities. The ratios of acetylation reactions to one another have been observed to change with low salt, high salt, and on binding of the various mAbs. The observed losses in enzymatic activity as the pI increased cannot be accounted for by proteolytic degradation because the molecular weight had not been changed. The AT was tested and found to be negative for carbohydrate, thus loss of an acidic glycan is not responsible for these differences in pI. Whether a loss of phosphate or sulfate groups could account for the higher pI values has not yet been explored. It is also possible that the increase in pI and loss of activity could be due to conformational differences. The microheterogeniety observed via pI determinations and differences in acetylation capabilities appears to be an artifact of the purification process, although it cannot be ruled out that there is a family of similarly sized and closely related enzymes that share antigenic sites and are also capable of these acetylation reactions. Alternative experimental approaches with molecular biologic techniques may provide further insight into this enzyme system that is believed to play an important role in modifying the carcinogenic effects of the aromatic amines.

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